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Reduced evolutionary rate in reemerged Ebola virus transmission chains

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On 29 June 2015, Liberia's respite from Ebola virus disease (EVD) was interrupted for the second time by a renewed outbreak ("flare-up") of seven confirmed cases. We demonstrate that, similar to the March 2015 flare-up associated with sexual transmission, this new flare-up was a reemergence of a Liberian transmission chain originating from a persistently infected source rather than a reintroduction from a reservoir or a neighboring country with active transmission. Although distinct, Ebola virus (EBOV) genomes from both flare-ups exhibit significantly low genetic divergence, indicating a reduced rate of EBOV evolution during persistent infection. Using this rate of change as a signature, we identified two additional EVD clusters that possibly arose from persistently infected sources. These findings highlight the risk of EVD flare-ups even after an outbreak is declared over.

INTRODUCTION

After a roughly 2-year-long struggle, all known chains of human-to-human Ebola virus disease (EVD) transmission have finally been stopped in Western Africa. However, the region remains under enhanced surveillance to rapidly detect any cases associated with a missed transmission chain or a reintroduction of the disease. As of 28 February 2016, 28,639 EVD cases had been reported by the World Health Organization (WHO), including 11,316 associated deaths. Liberia was the first of the three heavily affected Western African countries to interrupt active transmission of Ebola virus (EBOV) from symptomatic individuals (February 2015). Since then, the country has experienced three renewed outbreaks ("flare-ups") of EVD. The first occurred in March 2015, resulting in one confirmed case; the second started in June 2015, resulting in seven confirmed cases; and the third occurred in November 2015, resulting in three confirmed cases. For all three flare-ups, no known symptomatic source could be identified, suggesting a deviation from the typical human-to-human transmission involving an acutely infected individual. The March 2015 case was ultimately shown to be associated with male-to-female sexual transmission from a persistently infected EVD survivor, demonstrating an additional mechanism for EBOV infection (1).

Here, we use epidemiological and genomic data to investigate the source of the second Liberian flare-up, centered in Margibi County during June to July 2015. We demonstrate that this second flare-up,

like the first, originated from a persistently infected source within Liberia. The viral genomes from the first and second flare-ups were characterized by significantly low levels of genetic divergence compared to other viral genomes sequenced from samples collected from infected persons in Liberia during the epidemic, indicating a reduced rate of EBOV evolution during persistent infections.

RESULTS

Liberia's second EVD flare-up occurred during June to July 2015

On 28 June 2015 (101 days after the previous confirmed case of EVD in Liberia), an oral swab was collected from a deceased 17-year-old male (from the village of Needowein) during routine EVD surveillance in Margibi County. One day later, the sample tested positive for EBOV RNA by quantitative reverse transcription polymerase chain reaction (qRT-PCR). From 30 June to 12 July, seven additional epidemiologically linked individuals with current or recent EVD-like clinical signs were identified. Each of the seven patients was transported to an Ebola (virus disease) treatment unit. Six patients tested positive for EBOV RNA via qRT-PCR, and one of these individuals subsequently died. The seventh patient's tests were indeterminate (positive with one assay and negative with another). The surviving patients and their families, friends, and contacts were interviewed about recent activities, including travel to neighboring countries with ongoing EBOV transmission, hosting of visitors, funeral attendance, sexual contact with EVD survivors, and/or consumption of bats or nonhuman primates. However, no such activities were reported.

Flare-up represents reemergence of a Liberian transmission chain

We isolated and sequenced EBOV RNA directly from serum and oral swab samples. Coding-complete EBOV genomes [using nomenclature

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defined by Ladner *et al.* (2)] were assembled from eight samples from five patients in the Needowein flare-up (Table 1). All eight genome sequences were nearly identical, collectively differing at only three sites (positions 3171, 5219, and 18764; table S1). Phylogenetic comparison with other EBOV sequences from Western Africa indicated that the Needowein flare-up was a continuation of the Western African EVD outbreak that began in 2013 and was not caused by an independent introduction from an unknown nonhuman reservoir (Fig. 1A). Viral sequences from these five patients shared eight derived substitutions with other sequences from Western Africa (compared to the first three genomes from the Western African outbreak; GenBank: KJ660346, KJ660347, and KJ660348) (3), and each viral genome had at least one additional substitution unique to the Needowein flare-up (position 10130; table S1). More specifically, the new sequences fell within a predominantly Liberian sublineage (LB2) derived from the SL2 lineage (4, 5) (Fig. 1). The SL2 lineage is distinct from the lineages that were circulating in Guinea and Sierra Leone (GN1, SL3, and SL4; Fig. 1A) (6, 7), the only two countries with active EBOV transmission during and just before the Needowein flare-up. Therefore, it is unlikely that the Needowein flare-up was caused by the reintroduction of EBOV into Liberia from a neighboring country. Rather, this cluster appears to have originated from the reemergence of a virus from a previous Liberian EBOV transmission chain.

Flare-up was linked to the Barclay Farm EVD cluster during August to September 2014

The epidemiological investigation revealed that multiple relatives of two patients (LIBR4808/4866 and LIBR5046) in the Needowein flare-up previously shared a home in the nearby community of Barclay Farm. Nine of the 22 occupants of the Barclay Farm household had confirmed EVD during August to September 2014; eight died, and a 13-year-old male was the only known EVD survivor (Table 1). Interviews revealed

that an adult female from the household, who also resided part-time in Needowein, had experienced clinical symptoms including body aches and vision problems in the weeks after caring for her mother, who died at home of confirmed EVD in August 2014. The adult female self-treated with unspecified traditional medicine but did not visit a health care facility or have a sample taken. She also reported having a miscarriage following her EVD-like symptoms. By October 2014, she and at least two relatives relocated permanently from Barclay Farm to Needowein, where they shared a home with the two Needowein patients (LIBR4808/4866 and LIBR5046). The 13-year-old survivor from Barclay Farm (LIBR20004) also moved to Needowein several months later.

To explore a potential connection between the 2014 Barclay Farm cluster and the 2015 Needowein flare-up, we located frozen serum samples taken during August to September 2014 from six of the nine Barclay Farm family members with confirmed EVD (Table 1). Genomic EBOV sequencing revealed that two of the decedents (LIBR20002 and LIBR20005) had EBOV genomes that differed by only one substitution from those in the Needowein flare-up (Fig. 1B and table S1). The other four genomes, which included the sequence recovered from the surviving 13-year-old male (LIBR20004), proved to not be a component of the Needowein transmission chain, excluding the boy as a potential EBOV source (Fig. 1B).

The investigation also revealed that five of the initial Needowein patients, as well as other community members, had butchered and consumed a dog that relocated with the family from Barclay Farm, after it had died on 10 June 2015 (estimated date). However, remains from the dog carcass and a deceased puppy tested negative for EBOV RNA.

Genomes from flare-up exhibit reduced evolutionary rate
The level of sequence divergence between the Needowein EBOV genomes and the inferred root of the Liberian portion of the Western African EVD outbreak was significantly lower than expected, given the

Table 1. Patient information for sequenced samples ordered by date tested. F, female; M, male; ND, not determined.

Sample ID	Sex	Age	Status	Village	County	Sample type	Date tested	Cluster	GenBank accession number
LIBR20001	F	ND	Deceased	Barclay Farm	Margibi	Blood	10 August 2014	Barclay Farm	KU220277
LIBR20002	M	50	Deceased	Barclay Farm	Margibi	Blood	24 August 2014	Barclay Farm	KU220278
LIBR20003	F	20	Deceased	Barclay Farm	Margibi	Blood	24 August 2014	Barclay Farm	KU220279
LIBR20004	M	13	Alive	Barclay Farm	Margibi	Blood	24 August 2014	Barclay Farm	KU220280
LIBR20005	F	4	Deceased	Barclay Farm	Margibi	Blood	25 August 2014	Barclay Farm	KU220281
LIBR20006	F	28	Deceased	Barclay Farm	Margibi	Blood	25 August 2014	Barclay Farm	KU220282
LIBR4748	M	17	Deceased	Needowein	Margibi	Oral swab	29 June 2015	Needowein	KU220269
LIBR4783						Blood*	30 June 2015		KU220270
LIBR4808	M	23	Alive	Needowein	Margibi	Blood	1 July 2015	Needowein	KU220271
LIBR4866						Blood	2 July 2015		KU220272
LIBR5046	M	40	Alive	Needowein	Margibi	Blood	7 July 2015	Needowein	KU220273
LIBR5047	F	13	Alive	Needowein	Margibi	Blood	7 July 2015	Needowein	KU220274
LIBR5081						Blood	8 July 2015		KU220275
LIBR5307	F	22	Deceased	Paynesville	Montserrado	Blood	12 July 2015	Needowein	KU220276

*Postmortem heart puncture.

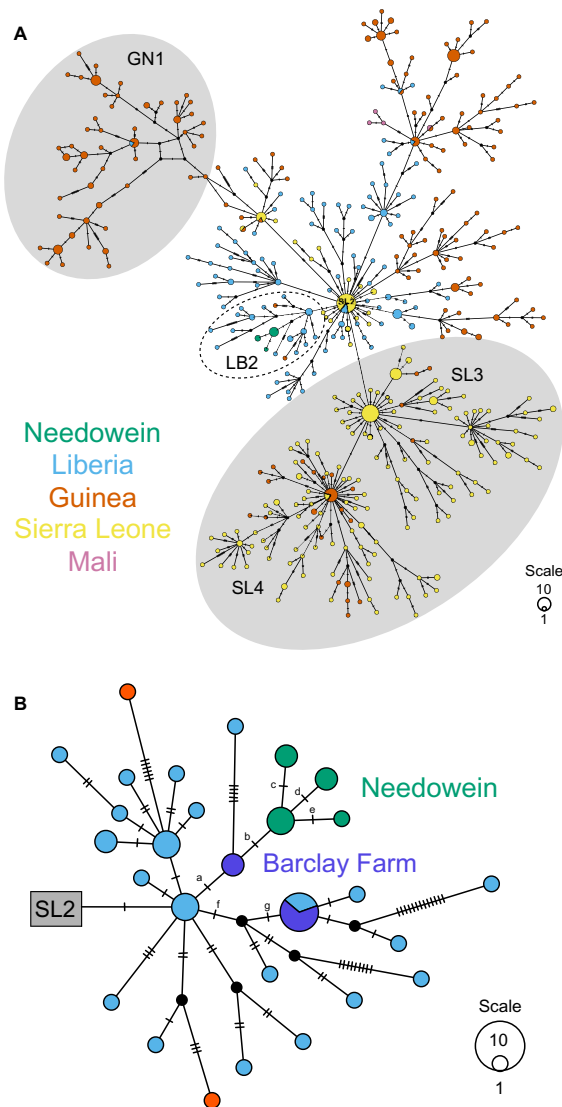


Fig. 1. The Needowein flare-up represents the reemergence of an EBOV transmission chain from the Liberian portion of the Western African EVD outbreak. (A) Median-joining haplotype network constructed from a full genome alignment of 583 Western African EBOV sequences (see table S3 for accession numbers). The dashed oval indicates the LB2 sublineage (SL2 lineage), which includes the sequences from the Needowein flare-up. Gray shading indicates the lineages that were circulating during February to October 2015 in Sierra Leone and Guinea (GN1, SL3, and SL4). (B) Median-joining haplotype network depicting the LB2 sublineage (see table S3 for accession numbers), including six sequenced genomes from the Barclay Farm cluster during August to September 2014 (purple). See table S1 for a description of the labeled substitutions (a to g).

timing of the Needowein flare-up (Fig. 2). On the basis of patterns of sequence divergence throughout the Liberian EVD epidemic, we estimated an expected sampling date of 11 September 2014 [95% highest posterior density (HPD) interval, 26 July to 9 November 2014] for the initial Needowein case (Fig. 2B). The difference of more than 9 months between the observed (28 June 2015) and estimated sampling dates is equivalent to a roughly 27-fold decrease in the

average rate of substitution on the branch leading to the Needowein flare-up. We also observed a fivefold reduction in the evolutionary rate associated with male-to-female sexual transmission of EBOV in March 2015 (Fig. 2B) (1). In that case, the male survivor had first reported EVD symptoms on 9 September 2014, approximately 6 months before the sexual transmission event (1). A plasma sample was collected from the female patient during her acute infection (20 March 2015), and the assembled EBOV genome (GenBank: KT587343) exhibited sequence divergence, relative to other Liberian EBOV sequences, consistent with a sampling date approximately 5 months earlier (12 October 2014; 95% HPD interval, 23 August to 4 December 2014).

The absence of active Liberian transmission chains before the Needowein flare-up and the case of sexual transmission led us to consider persistently infected survivors as a possible EBOV source for these flare-ups. However, transmission from EVD survivors may have also contributed earlier in the epidemic. Using the signature of a reduced evolutionary rate, we identified other EVD cases possibly linked to persistently infected individuals. Root-to-tip analysis identified 15 additional EBOV genomes (table S2) as outliers with lower-than-expected sequence divergence (95% prediction interval; Fig. 2A). Among these outliers were four LB7 sublineage sequences from a Liberian EVD cluster that occurred during December 2014 to February 2015, centered in the St. Paul Bridge community in Monrovia, Montserrado County (8). Other EVD cases were reported in the same area around the time of the index patient's onset of symptoms, and the index patient had recently traveled to Grand Cape Mount County, where there was ongoing human-to-human transmission of EBOV. However, the family of the index patient reported no known contact with other EVD patients, and genomic surveillance of samples collected during this period has not identified other active EBOV transmission chains with LB7 sublineage viruses (fig. S1A). Also among the 15 outliers were six Guinean EBOV genomes of the LB4 sublineage (5, 9). We were unable to obtain epidemiological data for these cases, but their timing (1 December 2014 to 27 January 2015), collection sites (neighboring Faranah and Kissidougou Prefectures in Faranah Region), and sequenced EBOV genomes (Fig. 2B and fig. S1B) indicate a potential epidemiological link. It is unclear whether similar links exist between any of the other five outliers (table S2); one has the basal SL2 haplotype, two are from the LB2 sublineage, and two are from the LB7 sublineage.

DISCUSSION

The cluster of EVD cases that occurred in and around Needowein village during June to July 2015 was the second EVD flare-up in Liberia following the successful control of the Liberian outbreak (that is, multiple weeks with no new cases). The first Liberian EVD flare-up occurred in March 2015 in Montserrado County. This first flare-up has been attributed to sexual transmission from a persistently and subclinically infected male Liberian EVD survivor and was limited to a single case (1). Although larger in scale, our genetic analysis has indicated that the Needowein flare-up also represents the reemergence of a Liberian EBOV transmission chain. Given the ongoing surveillance in Liberia during this period and the absence of an EVD case in Liberia for 3 months before this flare-up, this finding indicates that the Needowein flare-up originated from a persistently infected source.

We established both epidemiological and genetic links between the Needowein flare-up and a cluster of EVD cases that occurred nearly

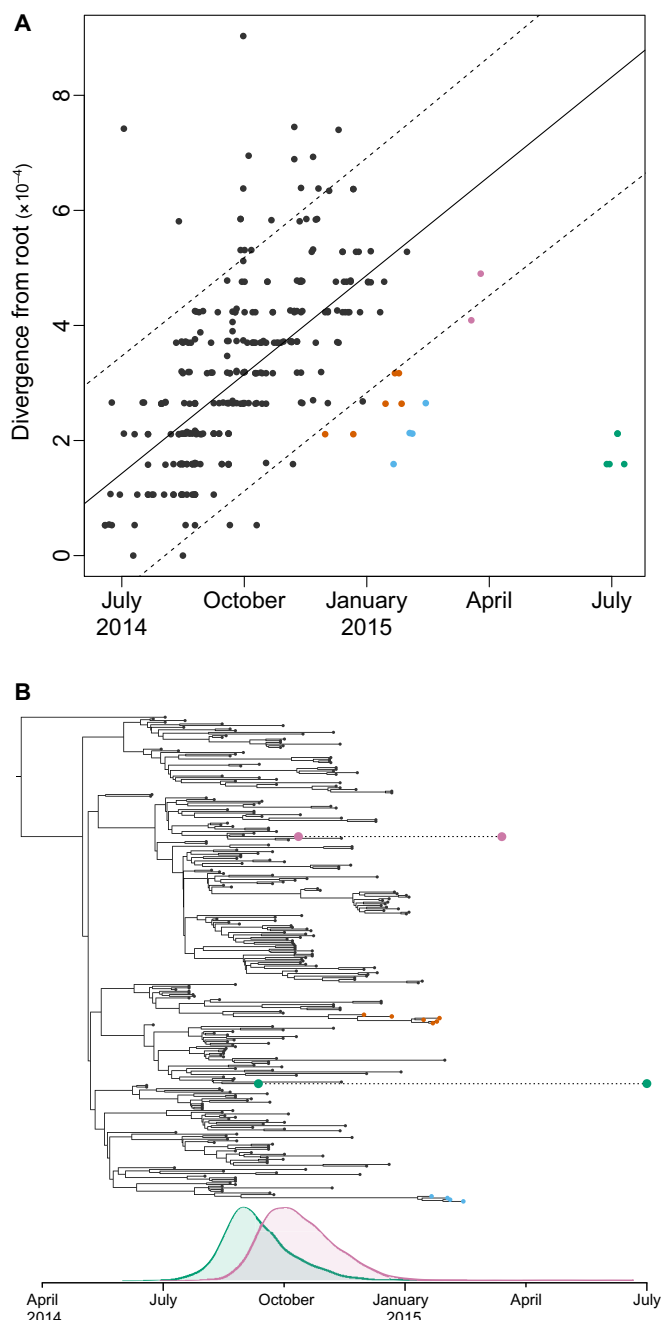


Fig. 2. Several EVD case clusters, including the Needowein flare-up (June to July 2015), are characterized by EBOV with lower-than-expected sequence divergence based on sampling date. (A) Root-to-tip distance versus sampling date for the Liberian portion of the SL2 lineage, including sequences from Guinea and Mali linked to reintroductions from Liberia (5). Green, Needowein flare-up; pink, sexual transmission (1); blue, St. Paul Bridge cluster (8); orange, Guinean cluster; dashed lines, 95% prediction interval. (B) Temporal maximum clade credibility tree from BEAST (Bayesian evolutionary analysis by sampling trees). Circles represent sampled EBOV genomes colored as described in (A). For the Needowein flare-up (green) and the March 2015 case of sexual transmission (pink), the dashed lines represent the difference between the actual date of sampling (right) and the estimated sampling date from the BEAST analysis (left). The posterior distributions for the estimated sampling dates are shown below the tree.

1 year earlier in the nearby Barclay Farm community. However, we were not able to definitively identify the persistently infected source for the Needowein flare-up. We assembled two EBOV genomes from Barclay Farm patients (LIBR20002 and LIBR20005) that differed from those obtained during the Needowein flare-up by only one nucleotide. Such genetic similarity is consistent with direct transmission (1); however, these two Barclay Farm patients had died in August 2014. The woman from Barclay Farm, who relocated to Needowein, is a potential source. However, her presumed EBOV infection was never confirmed, and EBOV transmission from a persistently infected female survivor has not yet been documented. The dog, which was also relocated from Barclay Farm to Needowein, is another potential source. However, there is currently no conclusive evidence that dogs can even become infected with EBOV (10), let alone serve as intermediate hosts for human infection. Alternatively, the source might have been an unidentified survivor with an epidemiological link to the cluster of cases in Barclay Farm. Margibi County was heavily affected by EVD during August to September 2014, with an average of 74 confirmed and probable cases reported weekly (11).

The sexual transmission case (first flare-up), the Needowein cluster (second flare-up), and the outlier cases identified from earlier in the epidemic (table S2) indicate that reduced rates of EBOV evolution can occur under certain circumstances and that persistent, subclinical infection is likely one such circumstance. Similar reductions in evolutionary rate have been observed during persistent infections of human parvovirus B19 (12); however, the mechanisms responsible for such reductions are poorly understood, especially for RNA viruses. Currently, there are no known mechanisms for persistence in the absence of replication (that is, dormancy) for RNA viruses without a DNA stage. However, the observed reduction in the rate of evolution could be explained by a reduced rate of viral replication (13). After the acute phase of EVD, EBOV RNA can no longer be detected in blood, but the virus can persist in immune-privileged sites, such as the eye and testes (14, 15), where EBOV may replicate more slowly.

The primary goal of this investigation was to quickly interrupt transmission of EVD and prevent further spread. This goal was accomplished, and on 3 September 2015, Liberia was again declared free of EVD (16). However, during 20 to 21 November 2015, three additional epidemiologically linked EVD cases were confirmed in Liberia. Genomic investigation into the source for these cases is ongoing, but given the time lapse since the last EVD case in Liberia, we speculate that this new cluster is also linked to the reemergence of a previous transmission chain. Although transmission from persistent infections appears to be relatively rare, persistent subclinical EBOV infections have been shown to occur in male EVD survivors (14), and single transmission events from persistent infections have the potential to reignite person-to-person EBOV transmission via the typical route (that is, via contact with body fluids of a symptomatic person). Fortunately, Liberia's rapid response to these postepidemic clusters has quickly interrupted transmission. Liberia, Sierra Leone, and Guinea will need to maintain heightened vigilance during the coming months, or perhaps longer, to rapidly contain any future flare-ups.

MATERIALS AND METHODS

Sample processing and sequencing

This investigation was conducted as part of the Ebola public health response in Liberia. It was not considered human subject research, in

accordance with the U.S. federal human subject protection regulations and the U.S. Centers for Disease Control and Prevention's Guidelines for Defining Public Health Research and Public Health Non-Research. Work conducted at the Liberian Institute for Biomedical Research (LIBR) was carried out with the consent of the National Incident Management System of the Ebola Virus Disease Outbreak and the Liberian Ministry of Health and Social Welfare, and was supervised by the LIBR institutional review board. All the information obtained from the participants was anonymized for this report.

RNA was extracted from serum samples using the QIAamp Viral RNA Mini Extraction Kit on a Qiagen EZ1 extraction robot and tested for the presence of EBOV RNA by the LIBR reference laboratory. Samples were prepared using a modified version of the Illumina RNA Access Kit as previously described (1). Samples were prepared multiple times and different fragmentation times were used to obtain optimal RNA sequencing libraries because of the inability to determine accurate size distribution of RNA on-site (table S4). Samples prepared for the 17 September 2015 sequencing run had a modification in the enrichment protocol (see table S4). Instead of pooling samples in groups of four before enrichment, we enriched all samples separately and used a quarter of the reagents for the enrichment. This was done to eliminate enrichment bias. Samples were sequenced on an Illumina MiSeq housed at the LIBR Genome Center (17).

The random hexamer associated with read 1 and the Illumina adapters were removed from the sequencing reads using Cutadapt version 1.21 (18), and low-quality reads and bases were filtered using PRINSEQ-lite version 0.20.4 (19). Reads were aligned to the reference using Bowtie 2 (20), duplicates were removed with Picard (<http://broadinstitute.github.io/picard>), and a new consensus was generated using a combination of SAMtools version 0.1.18 (21) and custom scripts. Only bases with a Phred quality score ≥ 20 were used in consensus calling, and a minimum of $3\times$ read-depth coverage, in support of the consensus, was required to make a call. Positions lacking this depth of coverage were treated as missing (that is, called "N").

Genomic analysis

PopART version 1.7.2 (<http://popart.otago.ac.nz>) was used to create haplotype networks using the median-joining reconstruction method. To place the eight Needowein flare-up sequences within the context of the full Western African outbreak, these sequences were compared to 575 representative EBOV genomes from Guinea, Liberia, Mali, and Sierra Leone (only sequences with $>99\%$ genome coverage; see table S3 for accession numbers). Because of missing data, 1389 sites (7.3% of total genome) were excluded from the analysis, including 89 sites with variability among samples (8.5% of all variable sites). This analysis indicated that the Needowein genomes belonged to the SL2 lineage, LB2 sublineage. To further refine the placement of these genomes within this sublineage, a subset analysis was conducted including the eight sequences from the Needowein flare-up, six sequences from the August to September 2014 EVD cluster in Barclay Farm, and all 29 published EBOV genomes from the LB2 sublineage (see table S3 for accession numbers). Because of missing data, 1834 sites (9.7% of total genome) were excluded from the analysis, including 10 sites with variability among samples (11.5% of all variable sites).

Path-O-Gen version 1.4 (22) was used to calculate root-to-tip distances using a maximum-likelihood phylogeny constructed with PhyML version 3.0 (23) with a general time-reversible model. This analysis was restricted to Liberian EBOV sequences from the SL2 lineage

with $\geq 93\%$ genome coverage, as well as EBOV sequences from Guinea and Mali resulting from reintroductions from Liberia (see table S3 for accession numbers) (5). Only one sequence (the earliest collected) was included from each of the Needowein flare-up patients. The phylogenetic tree was rooted using LIBR10089 (GenBank: KT725377), which is one of the two Liberian EBOV sequences identical to the basal SL2 haplotype (5). Robust regression was used to fit a linear model to the data, excluding the sequences from the Needowein flare-up and the sexual transmission case, both of which are associated with transmission from persistently infected sources.

Treating sampling date as a random variable, we used BEAST version 1.8.2 (22) to estimate expected sampling times for two Liberian EBOV genome sequences linked to transmission from persistently infected sources: the March 2015 case of sexual transmission (GenBank: KT587343) and the earliest genome from the June to July 2015 Needowein flare-up (GenBank: KU220269). Sites were partitioned into noncoding intergenic regions and codon positions 1, 2, and 3. The evolution of all four site partitions was modeled by independent HKY substitution models with Γ^4 -distributed rate heterogeneity. A relaxed molecular clock was used with lognormally distributed rate categories (24). The nonparametric Bayesian SkyGrid tree prior (25) was used. The prior on the mean of the lognormal distribution [$N(1.144 \times 10^{-3}, 5.7968 \times 10^{-3})$] was informed by previously estimated rates (5). Markov chain Monte Carlo was run for 100,000,000 iterations, sampling every 10,000 iterations. The first 10,000,000 iterations were discarded as burn-in.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/2/4/e1600378/DC1>

fig. S1. Median-joining haplotype networks depicting two clusters of EBOV genomes characterized by significantly low levels of genetic divergence (from the root of the Liberian portion of the EVD outbreak) relative to testing date (see Fig. 2A).

table S1. Substitutions that define the Needowein and Barclay Farm samples (see Fig. 1B).

table S2. EBOV genomes with lower-than-expected levels of sequence divergence given the testing date (see Fig. 2A).

table S3. EBOV genomes used in genetic analyses.

table S4. Sequencing statistics for samples processed for study.

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